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Filed : December 30, 2003

REMARKS

Definiteness

The Examiner has rejected Claim 1 under 35 U.S.C. 112, second paragraph, as being indefinite. Specifically, the Examiner has found unclear what structural limitation is placed on the method by the addition of the term "low density microarray" in Claim 1. The term "containing" was read by the Examiner as "comprising" and therefore, more than 3000 probes could be present on the array. Claim 1 has now been amended to recite "low density microarray consisting of capture probes for the detection of up to 1000 genes". Support for this amendment may be found in section [0056] and table 1 of the present specification.

The specification gives a clear teaching concerning the intended dimension of the low density array. See e.g., paragraph [0064] of the specification, wherein is set forth that the microarray "may contain capture probes for the detection of up to about 3000 different genes" and paragraph [0091] of the specification wherein is stated that "a preferred solid support is a low density array" and that the "assays of the invention may determine the expression level of about 14, 50, 100, 400, 1000 or even 3000 genes". The meaning is that the number of capture probes is limited to the minimum for getting good results focusing on the ABC transporters.

Novelty

The Examiner has rejected Claims 1-3, 5, 6, 10, 15 and 15 under 35 USC §102(b) as being allegedly anticipated by Watts et al. (2001 *J. Pharmacol. Exp. Ther.* 299:434-441).

To be anticipatory under 35 U.S.C. § 102, a reference must teach each and every element of the claimed invention. See *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379 (Fed. Cir. 1986). "Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. ...There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention." See *Scripps Clinic & Research Foundation v. Genentech, Inc.*, 927 F.2d 1565 (Fed. Cir. 1991).

Watts et al. describe the detection of multidrug resistance to doxorubicin. The multidrug resistant cell lines expressing MDR1 were compared with the respective sensitive cell line by isolating mRNAs, preparing cDNAs, which were then used as probes on the microarray. Figure 1 and Table 1 of Watts et al. lists several tested genes having, with the exception of MDR1 and ABC transporter 1, functions different from ABC transporters. Watts et al. discloses ABC A2, A3, A4, B1, B10, C2, C13, D3, and E1, but not all 49 human ABC transporters as shown in table

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1 of the present specification. Furthermore, Watts et al. uses a high-density microarray, as evidenced on page 435, right column under "Microarray fabrication", having probes to more than 5,000 genes on the array. Watts does not disclose a method employing a low-density microarray for the detection of up to 1,000 genes, comprising 49 human transporters selected from the ABC transporter subfamilies A, B, C, D, E, F and G for the determination of the resistance of cells versus the action of an active substance.

Therefore, currently amended claims 1-3, 5, 6, 10, 15 and 15 are novel over the cited reference, and their rejection under 35 USC §102(b) should be withdrawn.

Non-obviousness

The Examiner has rejected Claims 4, and 7-14 under 35 USC §103(a) as being allegedly unpatentable over Watts et al. (*J. Pharmacol. Exp. Ther.* 2001 299:434-441) as applied to Claims 1-3, 5, 6 15, and 16 above, and further in view of Nakayama et al. (*Int. J. Cancer* 2002 101:488-495); List et al. (*Blood* 1996 87:2464-2469); Dao et al. (*Human Mol. Genet.* 1998 7:597-608); and van den Heuvel-Eibrink et al. (*Int. J. Pharmacol. Ther.* 2000 38:94-110). Applicant respectfully disagrees.

As asserted above, Watts et al. do not teach or suggest all the claim limitations of the currently amended Claim 1. Watts does not teach quantification of 49 ABC transporters on a low density microarray consisting of capture probes for up to 1000 genes. The cited secondary references of Nakayama, List, Dao, and van den Heuvel-Eibrink fail to cure the deficiencies of the main reference. Nakayama teaches analysis of expression of 4 ABC transporters (MDR1, MRP1, MRP2 and BCRP) by PCR. Nakayama does not teach quantification of 49 ABC transporters on a low density microarray. List discloses detection of overexpression of protein, LRP, as indication for the outcome of acute myeloid leukemia, but does not teach quantification of 49 ABC transporters on a low density microarray. Dao discloses mouse and human versions of an imprinted gene, which is similar to bacterial and eukaryotic polyspecific metabolite transporters and multidrug resistance pumps, but also fails to teach quantification of 49 ABC transporters on a low density microarray. Van den Heuvel-Eibrink discloses the relevance of MDRs in the prognosis of leukemia and mentions Pgp, MRP1 to 6, LRP/MVP and BCRP; but it also fails to teach or suggest quantification of 49 ABC transporters on a low density microarray.

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Therefore, the combinations of the cited references do not teach or suggest all the claim limitations, and the rejection of Claims 4, and 7-14 under 35 USC §103(a) should be withdrawn.

The Examiner has rejected Claims 1-3, 5-6, 10 and 15-16 under 35 U.S.C. 103(a) as being unpatentable over Wang et al. (*Chinese J. Cancer Res.* 2002, 14(1):5-10) in view of Anereau et al. (*Proc. Amer. Assoc. Cancer Res.*, 2003, vol. 44, 2nd ed, abstract #3992, p. 796-797). Specifically, the Examiner stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have incorporated the array disclosed by Annereau into the analysis of gene expression changes in resistant cells treated with an anti-cancer drug as disclosed by Wang to arrive at the claimed invention with a reasonable expectation for success.

Wang et al. describes gene expression profiling in multidrug resistant KB cells using high density microarrays (having 12,720 PCR products), and specifically mentions overexpression of MDR1 and MRPs. Annereau et al. describe using a high-density microarray (over 18,000 probes) to detect the expression of 36 members of the ABC-transporter superfamily in drug resistant cells. The combination of these references does not teach or suggest using a low-density microarray (no more than 1,000 probes) having capture probes specific for 49 ABC-transporters from subfamilies A-G.

Therefore, Claims 1-3, 5-6, 10 and 15-16 are non-obvious over the combination of the cited references, and their rejection under 35 USC §103(a) should be withdrawn.

The Examiner has rejected Claims 1-3, 6 and 15 under 35 U.S.C. 103(a) as being unpatentable over Lee et al. (*J. Pharmaceut. Sci.* 2003, vol. 92:2152-2163) in view of Langmann et al. (*Clinical Chemistry*, 2003, vol. 49:230-238).

Lee et al. refer to a micro array which may comprise capture probes MRP 1, 2, 3 and 6, specific for MDR 1. Langmann et al. disclose an RT-PCR method for detection and quantification of 47 currently known members of the ABC-transporter superfamily. Langmann et al. mention on page 237, left col., 1st par. that the high specificity of the TaqMan primer and probe sets is especially useful when analyzing a complete superfamily, as in our case all ABC transporter genes. Accordingly, the skilled person is not encouraged by Langmann et al. to set up a low-density microarray for the specific detection and quantification of 49 human ABC

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transporters selected from the ABC transporter subfamilies A, B, C, D, E, F and G as a solution has been already provided by Langmann.

Therefore, Claims 1-3, 6 and 15 are non-obvious over the combination of the cited references, and their rejection under 35 USC §103(a) should be withdrawn.

The Examiner has rejected Claims 1-3, 5, 6, 10, 15 16 under 35 U.S.C. 103(a) as being unpatentable over Watts et al. (*J. Pharmacol. Exp. Ther.*, 2001, 299:434-441) in view of Zammatteo et al. (*Clinical Chemistry*, 2002, vol. 48, no. 1, p. 25-34). The Examiner has rejected Claims 4, 7-14 under 35 U.S.C. 103(a) as being unpatentable over Watts et al. (*J. Pharmacol. Exp. Ther.*, 2001, 299:434-441) in view of Zammatteo et al. (*Clinical Chemistry*, 2002, vol. 48, no. 1, p. 25-34) as applied to claims 1-3, 5, 6, 10, 15 and 16 above, and further in view of Nakayama et al. (*Int. J. Cancer*, 2002, vol. 101:488-495), or List et al. (*Blood*, 1996, 87: 2464-2469), or Dao et al. (*Human Molecular Genetics*, 1998, 7:597-608), or van den Heuvel-Eibrink et al. (*Int. J. Pharmacol. Ther.* 2000, 38:94-110).

Specifically, the Examiner stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have incorporated the low density microarray format taught by Zammatteo into the method of detection of ABC transporters in multi drug resistance analysis to arrive at the claimed invention with a reasonable expectation for success. While Zammatteo discloses the use of the low density microarrays for the detection of MAGE-A sequences specifically, for their role in tumor immunotherapy, the process of constructing and the process of analyzing the low-density microarray would be equally applicable and useful for the detection of ABC Transporter sequences or any other sequence of interest.

The problem underlying the presently claimed invention is the provision of a reliable tool for determining multi drug resistance (MDR) in a sample and the inclusion of other indicative markers as well, relevant for an explanation of the clinical resistance of cells to drug treatment (see paragraph [0014] of the specification). The solution provided encompasses a method for determining MDR employing capture probes for 49 human ABC transporters selected from the ABC transporter subfamilies A to G in a low density micro-array giving thereby a complete overview about the resistance profile of a cell sample with respect to ABC transporters. The first aim of the application is to identify a pattern of resistance genes and to be able to use such information in order to choose the correct drug for appropriate cancer treatment (see paragraphs [0002] and [0018]). The interest of the assay is the link between the expression on many ABC

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transporters and the clinical data. The final information requirement for the patient from which the cells are analyzed is to know if the cells are resistant to a particular drug and/or which are the drugs the cells will be sensitive to. The knowledge on the expression of many ABC transporters has to be obtained on a quantitative basis (see paragraph [0065]). The low density microarray of the present invention permits the quantification of the expression, while the limitation of the high-density microarrays is loose quantification of the genes, and detection of only highly differentially expressed genes (see paragraph [0015]). Indeed, further studies using microarrays according to the presently claimed invention confirmed very clearly the potential of the invention and the unexpected finding that such approach allows to reach. Use of the presently claimed invention has been made which shows the potential and the unexpected results obtained by such low density array for the screening of the ABC transporters. For example, Gillet et al. (cf. abstract of *Cancer Genomics and Proteomics*, 3:97-106, 2006, attached) has used such low density biochips on human breast cancer samples. The authors report to have found unexpectedly that many of the ABC transporters already expressed in such tumors even before any treatment. Such observation was new and can explain why such tumors develop resistance to chemotherapy since the resistance genes are already expressed. From the abstract may be derived that "A low-density DNA microarray was recently developed for analysis of 38 ABC-transporter genes and 3 other transporters. In the present pilot study, clinical samples from 16 breast cancer patients were tested. Of the 41 transporters analyzed, 10 were not or very seldom expressed while 23 were found to be expressed, sometimes at very high levels, in the majority of the tumors. Comparison of the treated and untreated tumors showed an unexpected similarity of results. The signal obtained on the capture probes for ABCC6/8/9 was however found to be higher in the treated samples. The microarray data were validated on 15 ABC transporters genes by real-time PCR. The present results showed that the expression of the majority of the ABC transporters is a clear feature of breast tumors whether treated or not."

A change of the expression of at least 5 ABC transporters by a certain factor as compared to a reference is indicative of the development/presence of resistance of said cells to the substrate.

The present inventors found that change in the expression of as few as 5 ABC transporters is indicative, since a change of one ABC transporter affects the expression of other ABC transporters belonging to the same family or to a different family. In other words, when a

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gene is activated, it affects the expression of other genes which are not necessarily closely related genes, such as genes belonging to a different subfamily. This view is also supported by recent studies of Efferth et al. (*Mol. Cancer Ther.* 5:1986-1994, 2006, enclosed), which showed that the same chips allow to discover an unknown correlation between the resistance of cells from the leukemia with the over expression of the ABC A2/A3 transporters. Assays with modulation of the activity of one ABC transporter show that the inhibition of one transporter was compensated by the increase of another one. As stated in the abstract: "This indicates that down-regulation of one ABC transporter was compensated by up-regulation of the other." Since the level of one transporter influences the level of other ones, it is essential to detect a maximum of transporters at the same time on the same sample in order to detect such possible compensation mechanism in order to be able to promote a correct treatment against the tumor cells.

The presently claimed invention is further based on the experimental data showing a CV, for replicate arrays, lower than 20% and a ratio between two arrays (test and control) significantly different from 1 when the ratio of the signal between the two arrays is higher than 1.5. The clinical relevance of the assay is to detect a variation as small as possible in order to be able to predict the building up of a resistance of a cell to a particular drug. However the variation in the level is dependent on the reproducibility of the assay. The variation in the ratio has to be statistically significant since otherwise no results of biological validity are obtained. The present inventors found in this respect that it is possible to develop microarrays for ABC transporters with a reproducibility of hybridization of lower than 20% from one array to the other. Such arrays allow to obtain ratios significantly different from 1 between a test and a reference sample when the ratios are higher than 1.5, whereas in the prior art, a threshold of 2 or even higher is necessary for the determination of statistically different ratios if the quality of the array or of the assay is lower. Such a quantitative array has been shown to be useful in detecting unknown ABC transporters involved in tumor resistance. This finding has been confirmed on the given genes in for example, Efferth et al. and Steinbach et al. (see, *Mol. Cancer Ther.* 5:1986-1994, 2006 and *Clin. Cancer Res.* 15:4357-4363, 2006, both enclosed).

Furthermore, a low density array overcomes the difficulties of common high density microarrays, such as the loss of quantification of genes and their prevalence to detect primarily the highly differentiated genes (see paragraph [0015]). Additionally, the present microarray

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allows fast and simultaneous detection/quantification of several genes and facilitates the overview of the subfamilies expression in the sample (cf. section [0063] of the specification).

The combination of the cited references does not teach all of the claim limitations, therefore, currently amended Claims 1-16 are non-obvious over the cited references, and their rejection under 35 USC §1039a0 should be withdrawn.

The Examiner has rejected Claims 1-3, 5-6, 10 and 15-16 under 35 U.S.C. 103(a) as being unpatentable over Wang et al. (*Chinese J. Cancer Res.* 2002, 14(1):5-10) in view of Annereau et al. (*Proc. Amer. Assoc. Cancer Res.*, 2003, vol. 44, 2nd ed, abstract #3992, p. 796-797) and further in view of Zammattéo et al. (*Clinical Chemistry*, 2002, vol. 48, no. 1, p. 25-34). Specifically, the Examiner stated that would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have incorporated the array disclosed by Annereau into the analysis of gene expression changes in resistant cells treated with an anti-cancer drug as disclosed by Wang to arrive at the claimed invention with a reasonable expectation for success.

As asserted above, Wang et al. describes gene expression profiling in multidrug resistant KB cells using high density microarrays (having 12,720 PCR products), and specifically mentions overexpression of MDR1 and MRPs. Annereau et al. describe using a high-density microarray (over 18,000 probes) to detect the expression of 36 members of the ABC-transporter superfamily in drug resistant cells. The combination of these references does not teach or suggest using a low-density microarray (no more than 1,000 probes) having capture probes specific for 49 ABC-transporters from subfamilies A-G. Zammattéo discloses low density microarrays for the detection of MAGE-A sequences. However, the combination of these references does not teach all the claim limitations of the method for using a low-density microarray (no more than 1,000 probes) having capture probes specific for 49 ABC-transporters from subfamilies A-G. Therefore, Claims 1-3, 5-6, 10 and 15-16 are non-obvious over the cited references, and their rejection under 35 USC §103(a) should be withdrawn.

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CONCLUSION

Applicants have endeavored to address all of the Examiner's concerns as expressed in the outstanding Office Action. Accordingly, amendments to the claims, the reasons therefor, and arguments in support of the patentability of the pending claim set are presented above. In light of the above amendments and remarks, reconsideration and withdrawal of the outstanding rejections is specifically requested. If the Examiner finds any remaining impediment to the prompt allowance of these claims that could be clarified with a telephone conference, the Examiner is respectfully requested to initiate the same with the undersigned.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

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ABCA3 as a Possible Cause of Drug Resistance in Childhood Acute Myeloid Leukemia

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Abstract **Background:** A major issue in the treatment of acute myeloid leukemia (AML) is resistance to chemotherapeutic drugs. Multidrug resistance can be caused by ATP-binding cassette (ABC) transporters that function as drug efflux pumps. The majority of these proteins have not yet been examined in malignant diseases.

Experimental Design: A newly developed microarray for the simultaneous quantification of 38 ABC transporter genes and Taqman real-time PCR was used to analyze the expression of ABC transporters in pediatric AML and healthy bone marrow. Small interfering RNA was used to verify the role of ABCA3 in drug resistance.

Results: Using the microarray, we identified four new ABC transporters, which were overexpressed in many AML samples compared with healthy bone marrow: *ABCA2*, *ABCA3*, *ABCB2*, and *ABCC10*. The overexpression of these four genes was verified by real-time PCR in 42 samples from children with AML and 18 samples of healthy bone marrow. The median expression of *ABCA3* was three times higher in 21 patients who had failed to achieve remission after the first course of chemotherapy than in a well-matched group of 21 patients who had achieved remission at this stage ($P = 0.023$). Incubation of cell lines with a number of different cytostatic drugs induced an up-regulation of *ABCA3*. Down-regulation of *ABCA3* by small interfering RNA sensitized cells to doxorubicin.

Conclusion: Our results show that *ABCA2*, *ABCA3*, *ABCB2*, and *ABCC10* are overexpressed in childhood AML compared with healthy bone marrow. *ABCA3* is the most likely transporter to cause drug resistance.

A major issue in the treatment of acute myeloid leukemia (AML) is resistance to chemotherapeutic drugs. Many patients fail to respond to chemotherapy, and others relapse with resistant disease. Even with aggressive therapy, the survival rate in children with AML is only about 50% (1–3).

Several mechanisms of drug resistance have been identified. One of these is the overexpression of ATP-binding cassette (ABC) transporters that function as drug efflux pumps (4–6).

The family of ABC transporters consists of >40 different proteins. The function of many members of this family has not yet been investigated (7). The best-characterized ABC trans-

porter is the permeability P-glycoprotein, which is encoded by the multidrug resistance gene 1 (*MDR1* or *ABCB1*). Expression of P-glycoprotein/MDR1 has been identified as an independent adverse prognostic factor for complete remission and survival in adult patients with AML (4–6). Some studies suggested that it might be possible to improve the treatment of adults with AML by combining chemotherapy with drugs that inhibit the function of P-glycoprotein (8, 9). However, the clinical relevance of P-glycoprotein is much smaller in children with AML (10, 11).

It becomes more and more evident that a number of transporters act in concert to cause drug resistance. We could recently show that the expression of the breast cancer resistance protein (BCRP or *ABCG2*) and the expression of the MDR-associated protein 3 (MRP3 or *ABCC3*) are associated with a poor response (PR) to chemotherapy in children with AML (12, 13). Benderra et al. found the same results in adult patients with AML (14, 15). MRP3 is also associated with drug resistance in childhood ALL (16, 17).

The aim of this study was to investigate whether there are additional ABC transporters that are expressed in AML and involved in drug resistance. We were particularly interested in those ABC transporters that are overexpressed in AML samples compared with normal bone marrow because this might be an important prerequisite for using such proteins as therapeutic targets.

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Note: D. Steinbach and J.-P. Gillet contributed equally to this work.

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Patients and Methods

Patients and diagnosis. Pretreatment samples from 42 selected children with primary AML were analyzed. All of these patients had received full remission induction chemotherapy, and the remission status of all patients was analyzed before the next phase of therapy. Half of the patients showed a good response (GR); that is, they had achieved remission at this stage. The other 21 patients showed a PR; that is, they had not achieved remission after induction therapy. The patient characteristics of both groups are summarized in Table 1.

We used all samples from PR patients that were available. The 21 GR patients were selected from a total group of 35 GR patients. The selection criterion was to find the subgroup of GR patients that was the best possible match for the PR group with respect to the patient characteristics (Table 1). In doing so, we were able to compare two groups of patients with similar clinical features but a clearly defined difference in response to chemotherapy (i.e., *in vivo* drug resistance).

The patients were treated according to four multicenter studies: AML-I/82 (12 patients: 7 PR and 5 GR), AML-II/87 (13 patients: 7 PR and 6 GR), AML-BFM-93 (7 patients: 4 PR and 3 GR), and AML-BFM-98 (10 patients: 3 PR and 7 GR). All studies included induction therapies based on cytosine-arabioside and anthracyclines (18). The cumulative dosages in the induction therapies were AML-I/82 (100 mg/m² daunorubicin, 640 mg/m² cytarabine, 6 mg/m² vincristine), AML-II/87 (120 mg/m² daunorubicin, 1,050 mg/m² cytarabine, 450 mg/m² etoposide), AML-BFM-93 (180 mg/m² daunorubicin, 1,400 mg/m² cytarabine, 450 mg/m² etoposide), and AML-BFM-98 (36 mg/m² daunorubicin, 1,400 mg/m² cytarabine, 450 mg/m² etoposide).

The initial diagnosis of AML was determined by standard methods (1, 18). Written consent was given for the use of all patient samples for this study.

All patients were treated and diagnosed at the University Children's Hospital Jena. The samples were taken to the lab immediately.

Healthy donors. Eighteen samples of bone marrow were obtained from healthy adults (ages 19-41 years; 11 female and 8 male) who donated for bone marrow transplantation. Written consent was given for the use of these samples for this study. The samples were processed exactly in the same way as the patient samples.

Processing of samples. Ficoll-Hypaque density gradient centrifugation was done in all peripheral blood and bone marrow samples from patients and healthy controls. After this procedure, the percentage of leukemic cells in the AML samples was >90% as determined by May-Gruenwald-Giemsa-stained cytopins. Isolation of total RNA and transcription into cDNA were done as described (12, 13).

Expression profiling of ABC transporters with the DualChip human ABC. A detailed description of the DualChip human ABC, its validation, the procedure protocols, and the statistical analysis was given recently (19). In short, the chip is composed of single-strand DNA probes attached to a glass support by a covalent link. Each DNA probe is present in triplicates. The chip contains probes for 38 ABC transporters, eight housekeeping genes, and a number of positive and negative hybridization and detection controls. Because of the high homology between the ABC transporters, five capture probes are complementary to two or three closely related genes (*ABCA2/ABCA3, ABCB1/ABCB4, ABCG6/ABCG8/ABCG9*, and *Kir 6.1/Kir 6.2*). The total RNA was extracted using the Trizol method (Life Technologies, Gaithersburg, MD), and the reverse transcription was done from 10 µg of total RNA using the Superscript II enzyme (Invitrogen, Paisley, United Kingdom) before being hybridized on the array without amplification. After hybridization, the detection was done using a Cy3-conjugated anti-biotin IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The arrays were scanned with the ScanArray 4000 XL laser confocal scanner (Applied Biosystems, Foster City, CA) at a resolution of 10 µm. To maximize the dynamic range of microarrays, the same arrays were scanned using different photomultiplier tube settings. The use of different scanning intensities allows the quantification of both the high

Table 1. Initial patient data in 42 children with AML: 21 with GR to therapy and 21 with PR to therapy

	GR	PR
No. patients	21	21
Sex (male/female)	10/11	10/11
Age, y (25th/50th/75th percentile)	3/10/15	4/11/15
WBC count in 10 ⁹ /L (25th/50th/75th percentile)	15/43/153	26/52/80
Percentage of leukemic cells in WBC (25th/50th/75th percentile)	55/66/83	49/80/87
Inversion inv(16) (positive/negative)	5/16	1/20
Translocation t(8/21) (positive/negative)	2/19	3/18
Translocation t(9/11) (positive/negative)	3/18	2/19
Liver ≥3cm below costal margin (yes/no)	11/10	10/11
Spleen ≥3cm below costal margin (yes/no)	7/14	8/13
Auer rods (positive/negative)	2/19	3/18
FAB type (no. patients)		
M1	4	3
M2	4	4
M4	7	7
M5	6	7

and low copy expressed genes. After image acquisition, the scanned 16-bit image was used to quantify the signal intensities with the ImaGene 4.1 software (BioDiscovery, Los Angeles, CA).

Quantitative real-time PCR. Quantitative PCR was done as described (17). The ABI Prism 7700 Sequence Detector and Pre-Developed Assay Reagents (Applied Biosystems, Weiterstadt, Germany) were used for the quantification of all genes. The expression of the resistance genes was standardized for the expression of two housekeeping genes, *β-2-microglobulin* and *Abelson gene 1*. Both genes were shown to be expressed with little variability in healthy hematologic cells and AML (20). The geometric mean of both standardizations was used for the calculation of the expression of the resistance genes.

Serial dilutions of cDNA of reference cell lines were used to generate standard curves. The reference cell lines were MCF7/CH1000 (ABCA2, ABCA3, and ABCC10) and KG-1 (ABCB2). The expression of each gene in each sample was analyzed in duplicate. The regression coefficients of the standard curves ranged between 0.994 and 0.999. The variation of the duplicate measurements was extremely small compared with the variation between different samples. In the few cases where there was a substantial difference between the two values, the sample was reanalyzed.

Cell culture. Human leukemic Jurkat and CCRF-CEM cell lines were seeded in RPMI 1640 supplemented with 10% FCS. Human osteosarcoma 143B cells were grown in DHG medium (DMEM supplemented with 4.5 g/L glucose; Invitrogen) and 10% FCS (Life Technologies).

All of the cells were incubated under standard culture condition (5% CO₂ and 37°C).

Drug treatment. To analyze the changes in the expression of ABCA3 after incubation with cytostatic drugs, Jurkat and CCRF-CEM were treated with doxorubicin, cytarabine, vinblastine, vincristine, and epirubicin. The concentrations used with Jurkat cells were 0.1, 1, 0.1, 0.003, 0.1 µg/mL, respectively, and with CCRF-CEM cells were 1, 0.1, 0.001, 0.003, and 1 µg/mL, respectively. These concentrations were selected by determining the LD₅₀ for each drug in both cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays.

Transfection of small interfering RNA. Small interfering RNA (siRNA) transfection experiments were done using double-stranded RNA synthesized by Dharmacon (Lafayette, CO). A nontargeting siRNA (Scramble, Eurogentec, Searing, Belgium) was used as control. Cells

were transfected with Dharmafect 1 (Dharmacon) according to the manufacturer's instructions. Transfection efficiency in cells plated on coverslips was determined using FITC-labeled siRNA and evaluated to 85% to 90% after 24 and 48 hours by cell counting using a confocal microscope (Leica). siRNA efficiency on ABCA3 expression was determined by quantitative reverse transcription-PCR.

The effect of ABCA3 expression disruption in chemotherapy-induced resistance was analyzed as follows: 143B cells were seeded in 12-well plates at 100,000 per well 24 hours before being transfected with Dharmafect 1 for 24 hours with 50 nmol/L ABCA3 siRNA or equivalent treatment with nontargeting siRNA (Scramble, Eurogentec). Twenty-four hours after transfection, media were refreshed, and cells were incubated with 0.3 μ g/mL doxorubicin. This concentration was selected by determining the LD₅₀ for doxorubicin in 143B cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. ABCA3 mRNA level and the effect of drug treatment and silencing combination on cell viability were measured by quantitative reverse transcription-PCR and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 48 hours after transfection.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were seeded on 24-well plates and treated with doxorubicin (0.3 μ g/mL) for 24 hours at 37°C. After drug treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma, Gillingham, United Kingdom) was added to each well and

incubated for 2 hours at 37°C before medium removal. DMSO was then added and mixed for 2 hours at 37°C. Absorbance was measured at 570 nm using a spectrophotometer (Ultramark, Bio-Rad, Richmond, CA).

Statistical methods. Because the levels of gene expression did not follow normal distributions, the correlation between the ABC transporters and other clinical features was investigated by means of Spearman's correlation coefficients. Gene expression in different groups of patients was compared using the Mann-Whitney test for two groups and the Kruskal-Wallis test for more than two groups. All calculations were done using the SPSS 11.0 program (SPSS, Inc., Chicago, IL).

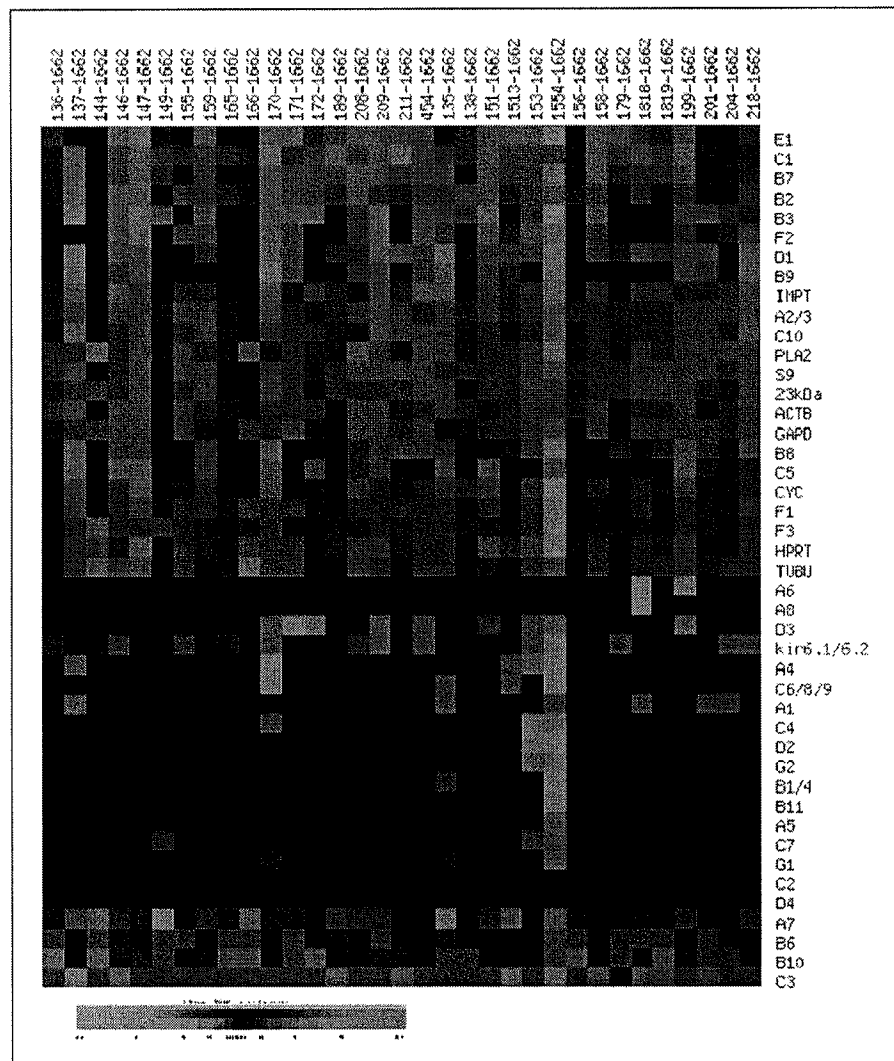
Cluster analysis. To investigate relationships between samples, we used the cluster program called EpClust (Expression Profiler, European Bioinformatics Institute, Cambridge, United Kingdom; <http://ep.ebi.ac.uk/EP>). We worked following the variables: hierarchical clustering, Manhattan distance, complete linkage (maximum distance).

Institutional review. This study was approved by the institutional review board of the University of Jena (#1545-05/05).

Results

Detection of overexpressed ABC transporters by expression profiling. Using microarrays, we aimed to identify ABC transporters that are overexpressed in AML samples compared with

Fig. 1. Expression of ABC transporters in 33 samples from AML patients compared with a sample of healthy bone marrow. Red squares, higher expression in the leukemic cells. Green squares, higher expression in the healthy control. Each column represents one patient, and each line represents one gene.



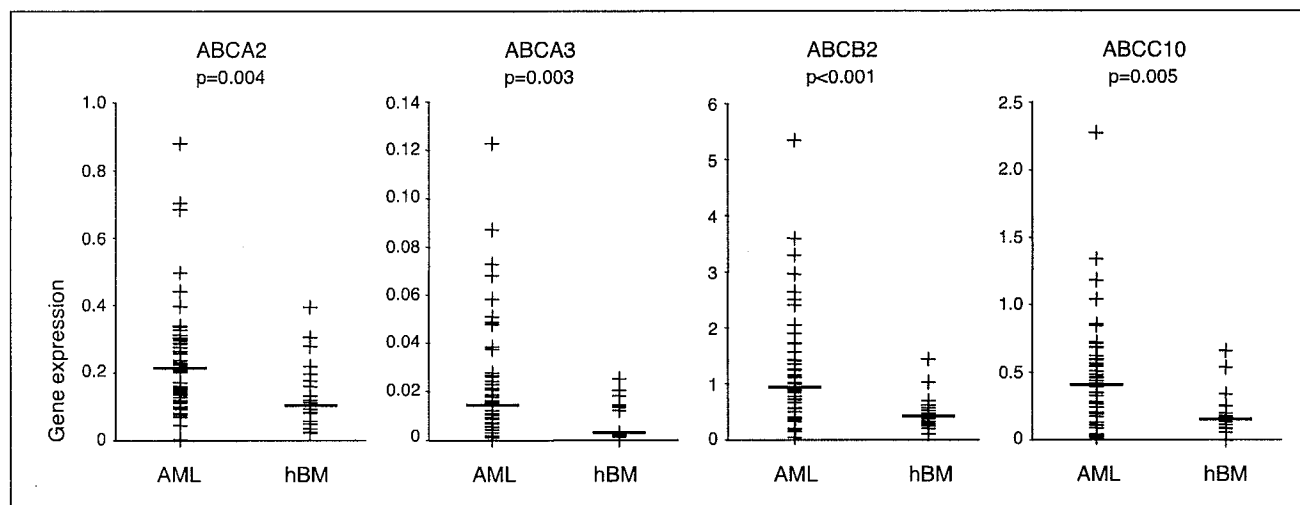


Fig. 2. Expression of four ABC transporters in samples from 42 children with AML and in 18 samples of healthy bone marrow (hBM). The median is indicated for each group. The expression is given in relation to the reference cell lines: MCF7/CH1000 (ABCA2, ABCA3, and ABCC10) and KG-1 (ABCB2).

healthy bone marrow. DualChip human ABC microarrays were done in samples from 33 patients (15 GR and 18 PR) and two samples of healthy bone marrow.

Many genes were overexpressed in a number of patient samples compared with healthy bone marrow. The five most consistently overexpressed genes were ABCA2/ABCA3 (ABCA2 and ABCA3 were detected by the same capture probe), ABCB2, ABCC1, and ABCC10.

In 22 patients, ABCA2/A3 was overexpressed >2-fold compared with both samples of healthy bone marrow. This was the case for ABCB2 in 24 patients, for ABCC1 in 24 patients, and for ABCC10 in 17 patients. The expression of all genes in all patient samples compared with one sample of healthy bone marrow is given in Fig. 1.

ABCC1 encodes the MRP1. Its expression in AML has already been studied intensively (4–6). The other four genes were selected for further analysis.

We also did an unsupervised hierarchical clustering with all genes and all patients. This analysis did not cluster the patients into the PR and the GR group, suggesting that the majority of ABC transporters do not have a major effect on drug resistance.

Analysis of overexpressed ABC transporters by real-time PCR. The expression of the selected genes was then analyzed by real-time PCR in all 42 patients and in 18 samples of healthy bone marrow. In accordance with the results of the microarrays, all four genes were overexpressed in many of the leukemic samples compared with the healthy controls. The median expression in the patient samples was two to four times higher, and the differences were highly significant (Fig. 2). Nevertheless, among the patients, there was a large variability in the expression of all four genes. The variation from the 25th percentile to the 75th percentile was 3-fold for ABCA2, 12-fold for ABCA3, 2-fold for ABCB2, and 5-fold for ABCC10.

Correlation with clinical features of AML. The four genes were investigated for their association with sex, age, FAB type, initial WBC, initial percentage of leukemic cells in WBC, and the presence of Auer rods and chromosomal aberrations t(8;21), t(9;11), and inv(16). The only associations that were statistically significant were a trend for higher levels of ABCA2

in patients with a higher percentage of leukemic cells in the initial WBC count ($P = 0.005$; Spearman's correlation coefficient = 0.47) a trend for lower levels of ABCA3 in older patients ($P = 0.041$; Spearman's correlation coefficient = -0.29), and a trend for higher levels of ABCC10 in patients with a higher initial WBC count ($P = 0.034$; Spearman's correlation coefficient = 0.31).

Correlation with response to remission induction therapy. An important question was whether any of the overexpressed ABC transporters were associated with PR to therapy because this would suggest that they are involved in drug resistance. The median expression of ABCA3 was about three times higher in the group of patients who did not achieve remission after

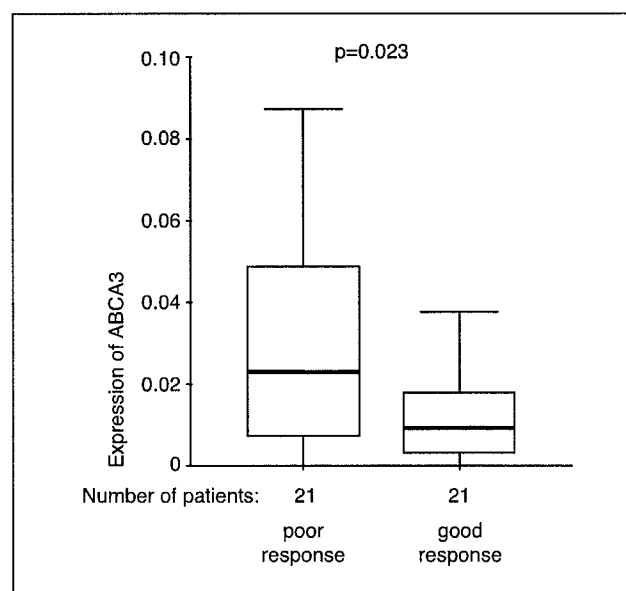


Fig. 3. Box plots (10th, 25th, 50th, 75th, and 90th percentile) of ABCA3 expression in patients who did not achieve remission after receiving full induction therapy (PR) and in patients who achieved remission after induction therapy (GR).

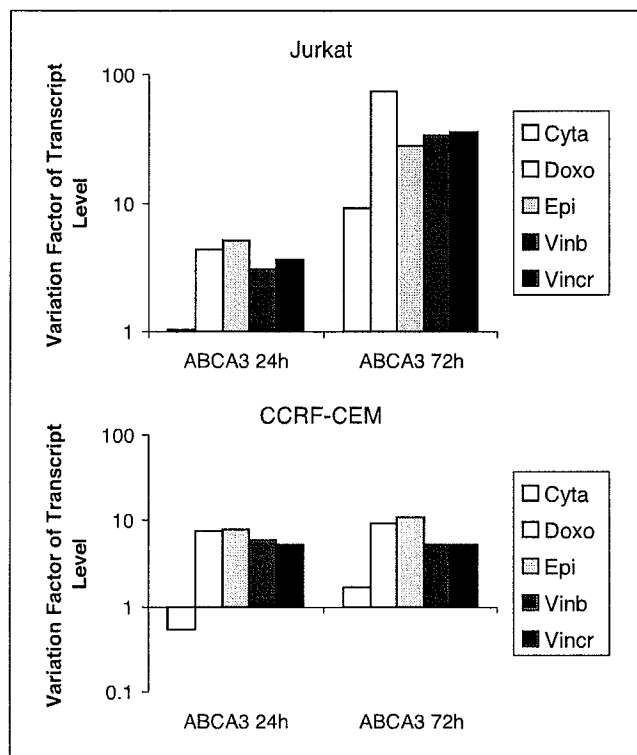


Fig. 4. Expression of ABCA3 in Jurkat and CCRF-CEM cells after treatment with cytostatic drugs. The expression after 24 and 72 hours is given in relation to the expression before treatment. Cyta, cytarabine; Doxo, doxorubicin; Epi, epirubicin; Vincb, vinblastine; Vincr, vincristine.

induction chemotherapy ($P = 0.023$; Fig. 3). When we compared PR and GR patients from each study separately, the expression of ABCA3 was always higher in the PR group. Therefore, this finding was not biased by the fact that the patients were treated according to four different multicenter studies with different induction therapies.

The PR and GR groups were well matched for the main clinical features of AML. The only important difference was a higher number of patients with the chromosomal aberration *inv(16)* in the GR group (Table 1). However, the expression of ABCA3 was not associated with this inversion.

None of the other three genes was associated with response to therapy.

Correlation of ABCA3 with previously analyzed ABC transporters. In the same group of patients who were analyzed in this study, we could recently show that ABCC3 (MRP3) and ABCG2 (BCRP) were associated with PR to therapy (12, 13). Neither ABCC3 nor ABCG2 were significantly correlated to the expression of ABCA3. In addition, the expression of ABCB1 (MDR1) was not significantly associated with the expression of ABCA3. In a partial correlation analysis with multivariate controlling for all of these three transporters, the association between ABCA3 and achievement of remission was still statistically significant ($P = 0.046$). Thus, ABCA3 was independently associated with response to therapy and not just coexpressed with other drug resistance genes.

Up-regulation of ABCA3 after treatment with cytostatic drugs. We first determined the LD₅₀ for each drug studied. The leukemic cell lines Jurkat and CCRF-CEM were then

incubated with five different cytostatic drugs. Each drug induced an up-regulation of the expression of ABCA3 (Fig. 4). This finding too suggests that ABCA3 might be involved in drug resistance. Either the cells show a higher expression as a reaction to the drugs or those cells, which already showed a high expression before the treatment, were more resistant and therefore survived the treatment.

Silencing of ABCA3 sensitizes cells to doxorubicin. Transfection in leukemic cell lines was done unsuccessfully. siRNA was then used to silence the expression of ABCA3 in the osteosarcoma cell line 143B. The level of the ABCA3 mRNA was quantified by real-time PCR. We found a down-regulation of 79% in the siRNA-transfected cells compared with the untransfected one (Fig. 5A). Anthracyclines are equally used in the treatment of osteosarcoma and AML. We, therefore, used doxorubicin to test whether the down-regulation of ABCA3 had a sensitizing effect on 143B cells. As shown in Fig. 5B, this was the case.

Discussion

Many studies have been carried out on the clinical relevance of ABC transporters. Thus far, all of these studies analyzed one or a small group of ABC transporters. For the most members of this family, the clinical relevance has still not been examined.

In this study, we used a newly developed low density DNA microarray for the simultaneous expression analysis of 38 ABC transporters. The advantage of a low-density microarray over

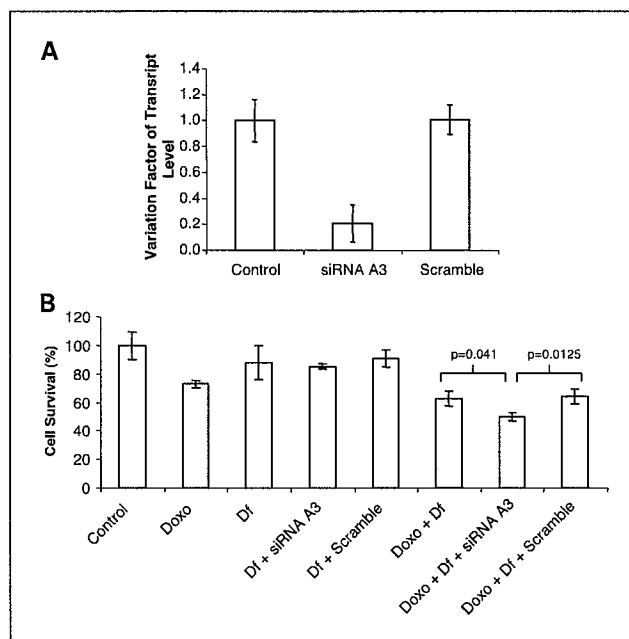


Fig. 5. A, variation factor of transcript level of ABCA3 gene measured by real-time PCR in 143B cells transfected with ABCA3 siRNA and nontargeting siRNA, respectively (Scramble). The results are given in relation to the expression in untreated samples (Control). B, sensitivity of 143B cells to doxorubicin after transfection with siRNA for ABCA3 and after transfection with nontargeting siRNA (Scramble). Cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The results are given in percentage of the cell survival in untreated samples (Control). Dharmatect (Dt) are the chemicals that were used for the transfection. P s were calculated by t tests.

high-density microarrays, which cover almost all known genes, is the good reproducibility and the specific validation that was done for the group of genes on the chip (19). This validation is particularly important in a group of genes that shows as much homology as the family of ABC transporters (21).

The aim of this study was to help to identify genes that cause drug resistance and hence can be used as therapeutic targets to sensitize resistant leukemic cells. In order not to sensitize normal hematopoietic cells at the same time, it is important to find those transporters that are expressed in leukemic cells but not or to a much lower degree in healthy bone marrow.

Using the microarrays, we could identify four new ABC transporters that were overexpressed in many AML patients compared with two samples of healthy bone marrow: ABCA2, ABCA3, ABCB2, and ABCC10. These findings were confirmed by real-time PCR in a larger group of patients and a much larger group of healthy controls (Fig. 2).

By using only two healthy controls for the microarrays and reserving the majority for the independent validation, we were at a slightly higher risk of missing interesting genes, but we could perform a more solid statistical analysis of the selected genes (Fig. 2).

We did not perform any purification of putative leukemic stem cells in the leukemic samples. Therefore, as healthy controls, we used bone marrow samples that were processed exactly in the same way as the leukemic samples with Ficoll-Hypaque density gradient centrifugation but no other selection process.

Of the four new ABC transporters that we found overexpressed in childhood AML, only ABCA3 was associated with PR to therapy ($P = 0.023$; Fig. 3). It is, therefore, the most likely candidate to cause drug resistance, but the prognostic effect of ABCA3 needs validation in a larger, nonselected, and homogeneously treated cohort of patients.

Furthermore, we could show that the incubation of leukemic cells with a number of different cytostatic drugs leads to a higher expression of ABCA3 (Fig. 4). Although this might also

be an unspecific stress reaction, this finding too points towards a role of ABCA3 in drug resistance.

143B cells could be sensitized to doxorubicin by silencing the expression of ABCA3 (Fig. 5B). The effect of silencing the expression of ABCA3 on cell survival was only 23% (Fig. 5B). This is not a major effect but, a down-regulation of mRNA does not lead to a full loss of the protein and its function. Therefore, the difference in cell survival is not a measure for the amount of drug resistance, which is caused by ABCA3. This amount is difficult to measure, but our clinical data suggest that it is relevant.

Recent results from other groups also suggested that ABCA3 could be involved in drug resistance. Yasui et al. (22) found that a number of drug-resistant cancer cell lines showed higher copy numbers of the ABCA3 gene and a stronger expression of the gene compared with the drug-sensitive parental cell lines. Hirschmann-Jax et al. showed that ABCA3 and BCRP are expressed in various malignant stem cells, and that the two genes together might be involved in the resistance of these cells against mitoxantrone (23).

Norwood et al. (24) described the expression of ABCA3 in an *in vivo* propagated human AML cell line. The same group could show that the protein ABCA3 was expressed in a panel of AML samples, and that the expression of the protein was strongly correlated to the expression of the gene (25). The latter finding is particularly important in combination with our data, showing that the expression of the gene is associated with response to therapy.

ABCA3 is located at intracellular membranes (26). It does not confer a "classic" drug efflux but rather seems to be involved in the intracellular sequestration and the vesicular transport of its physiologic substrates as well as chemotherapeutic agents, such as daunorubicin (25, 26).

Our results suggest that ABCA3 might be involved in drug resistance in AML. This finding warrants further studies on its physiologic role, the transport capacity, the clinical relevance, and the potential use as a therapeutic target to overcome drug resistance.

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Microarray Expression Profiling of ABC Transporters in Human Breast Cancer

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Abstract:

The ATP-binding cassette (ABC) transporters are highly conserved genes involved in the translocation of molecules through biological membranes. Several of them are involved in tumor drug resistance, and it is thought that many others may contribute to the development of the tumor phenotype in a still unknown way. A low-density DNA microarray was recently developed for the analysis of 38 ABC-transporter genes and 3 other transporters. In the present pilot study, clinical samples from 16 breast cancer patients were tested. Of the 41 transporters analyzed, 10 were not or very seldom expressed, while 23 were found to be expressed, sometimes at very high levels, in the majority of the tumors. Comparison of the treated and untreated tumors showed an unexpected similarity of results. The signal obtained on the capture probes for ABCC6/8/9 was, however, found to be higher in the treated samples. The microarray data were validated on 15 ABC-transporter genes by real-time PCR. The present results showed that the expression of the majority of the ABC transporters was a clear feature of breast tumors, whether treated or not.

Expression profiling of ATP-binding cassette transporters in childhood T-cell acute lymphoblastic leukemia

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Abstract

A major issue in the treatment of T-cell acute lymphoblastic leukemia (T-ALL) is resistance to chemotherapeutic drugs. Multidrug resistance can be caused by ATP-binding cassette (ABC) transporters. The majority of these proteins have not yet been examined in T-ALL. Using a newly developed microarray for the simultaneous quantification of 38 ABC transporter genes, we observed a consistent overexpression of *ABCA2/ABCA3* in clinical samples of ALL. Therefore, we analyzed the association of these two genes with drug resistance. Treatment of CCRF-CEM and Jurkat cells with methotrexate, vinblastine, or doxorubicin led to an induction of *ABCA3* expression, whereas a significant increase of *ABCA2* expression was only observed in Jurkat cells. To study the causal relationship of *ABCA2/3* overexpression with drug resistance, we applied RNA interference (RNAi) technology. RNAi specific for *ABCA2* or *ABCA3* led to a partial decrease of expression in these two ABC transporters. Upon cotreatment of RNAi for *ABCA2* with methotrexate and vinblastine, a partial decrease of *ABCA2* expression as well as a simultaneous increase of *ABCA3* expression was observed. Vice versa, *ABCA3* RNAi plus drugs decreased *ABCA3* and increased *ABCA2* expression. This indicates that down-regulation of one ABC transporter was compensated by the up-regulation of the other. Application of RNAi for both *ABCA2* and *ABCA3* resulted in a more efficient reduction of the expression

of both transporters. As a consequence, a significant sensitization of cells to cytostatic drugs was achieved. In conclusion, *ABCA2* and *ABCA3* are expressed in many T-ALL and contribute to drug resistance. [Mol Cancer Ther 2006;5(8):1986–94]

Introduction

Although cancer therapy for childhood T cell acute lymphoblastic leukemia (T-ALL) has remarkably improved during the past two decades, and normal life expectancy is a reality for many patients, a considerable number of patients cannot permanently be cured. In the majority of children suffering from T-ALL, remissions can be achieved by chemotherapy. Nevertheless, ~25% of the patients develop relapses. One major reason is the development of drug resistance. In patients with a T cell immunophenotype (T-ALL), drug resistance is even more commonly encountered.

The expression of one drug resistance gene, the multidrug resistance gene, *MDR1*, and its gene product, P-glycoprotein, has been well documented in leukemia. Its prognostic relevance for the development of drug resistance and worse outcome of patients has been shown for many tumor types including myeloid leukemia (1–3). In ALL, the relevance of the *MDR1*/P-glycoprotein is, however, still under debate. Although some authors found that high *MDR1*/P-glycoprotein expression and function is associated with the failure of chemotherapy and adverse prognosis (4–6), others have not (7–9). *MDR1*/P-glycoprotein belongs to the family of ATP-binding cassette (ABC) transporter, which comprises >40 different proteins (10). Other ABC transporters such as *ABCC1/MRP1*, *ABCC2/MRP2*, and *ABCG2/BCRP* also confer drug resistance in tumor cells. Their role in clinical treatment failure and worse outcome for patients with ALL is still a matter of discussion. Although there are other ABC transporters which also contribute to drug resistance of cancer cells (11), their role in ALL is still unknown.

The aim of this study was to examine whether additional ABC transporters are expressed in T-ALL and whether they are associated with drug resistance. A low-density microarray with 38 ABC transporter genes has recently been developed (12). We applied this microarray to clinical ALL samples in order to detect possible ABC transporters associated with the children's relapse, particular focus was drawn on *ABCA2* and *ABCA3*. Their role for drug resistance was then analyzed by experiments designed to induce gene expression after drug treatment, to decrease their expression, and to sensitize the cells. Finally, the microarray-based expression of *ABCA2* and *ABCA3* was correlated with the 50% inhibition concentration (IC₅₀) values of 60 cell lines for compounds of the Standard Drug Database of the National Cancer Institute, Bethesda, MD.⁷

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Note: T. Efferth and J.-P. Gillet contributed equally to this work.

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Materials and Methods

Cell Culture

The human T-ALL cell lines, CCRF-CEM and Jurkat, were maintained in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Life Technologies). Cells were passaged twice weekly. All experiments were done with cells in the logarithmic growth phase. The osteosarcoma cell line 143B was maintained in DHG medium (DMEM supplemented with 4.5 g/L glucose; Invitrogen, Paisley, United Kingdom) supplemented with 10% FCS (Life Technologies). Cells were passaged twice weekly. All of the cells were incubated under standard culture conditions (5% CO₂ and 37°C). The panel of 60 human tumor cell lines from the Developmental Therapeutics Program of the NCI has been described in detail previously (13).

Patients

Blood samples were obtained from 21 patients with T-ALL after obtaining informed consent. The main patient characteristics were: 14 males, 7 females; median age, 9.7 (1.8–16 years); median WBC count at presentation, 16.2 (8–450 Gpt/L); median percentage of leukemic cells in peripheral blood, 85% (18–99%). None of the patients were found positive for BCR/ABL translocation or MLL rearrangements. All samples were collected prior to the start of chemotherapy. The initial diagnosis of ALL was determined by Pappenheim-stained bone marrow smears and cytochemistry reactions (periodic acid-Schiff reaction, acid phosphatase, α -naphthyl acetate esterase, and myeloperoxidase reaction). Immunophenotype and chromosomal rearrangements were determined by standard methods (14). The patients were treated according to the multicentric Berlin-Frankfurt-Münster protocols (ALL-BFM-90, ALL-BFM-95, or ALL-BFM-2000). The main drugs used in all studies were steroids, methotrexate, cytosine-arabioside, anthracyclines, asparaginase, and vincristine (14, 15).

In vitro Response to Cytostatic Drugs

Jurkat and CCRF-CEM cell lines were treated for 72 hours at 37°C with doxorubicin, methotrexate, and vinblastine. The concentrations used with Jurkat cells were 0.1, 0.1, 0.1 μ g/mL, respectively, and with CCRF-CEM cells were 1, 1, and 0.001 μ g/mL, respectively.

After drug treatment, cells were centrifuged, placed in a 96-well plate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (Sigma, Gillingham, United Kingdom) was added to each well and incubated for 2 hours at 37°C before media removal. DMSO was then added and mixed for 2 hours at 37°C. Absorbance was measured at 570 nm using a spectrophotometer (Ultramark, Bio-Rad, Hercules, CA).

Expression Profiling of ABC Transporters with the DualChip Human ABC

A detailed description of the DualChip Human ABC, its validation, the procedure protocols, and the statistical analysis was given recently (12). The chip used the Xmer technology developed by Eppendorf (Hamburg, Germany)⁸ as proposed by ref. (16). Each DNA probe is present in triplicates. The chip contains probes for 38 ABC transporters, 8 housekeeping genes, and a number of positive and negative hybridization and detection controls (Fig. 1A). The total RNA was extracted using the Trizol method (Life Technologies) and reverse transcription was done from 10 μ g of total RNA using the Superscript II enzyme (Invitrogen) before being hybridized on the array without amplification.

To maximize the dynamic range of microarrays, the same arrays were scanned using different photomultiplier settings. The use of different intensities allows the quantitation of both the high and low copy expressed genes as described in ref. (17).

Real-time PCR

Real-time PCR followed a protocol described recently (12). For validation of microarray data, real-time reverse transcription-PCR was done on seven genes, i.e., *ABCA2*, *ABCA3*, *ABCA7*, *ABCC1*, *ABCC5*, *ABCF1*, and β -tubulin (housekeeping gene). The total RNA from four ALL samples (three patients with good response and one patient with poor response) taken randomly from the 21 ALL samples studied were used in the real-time reverse transcription-PCR ($n = 3$), and each reaction was done in duplicate. For measurement of mRNA expression of *ABCA2* and *ABCA3* in CCRF-CEM, Jurkat, and 143B cells, total RNA from three independent experiments were used in the real-time reverse transcription-PCR ($n = 3$), and each reaction was done in duplicate. A detailed procedure for calculating the relative expression ratio of a target gene in the test sample was reported elsewhere (18).

RNA Interference Technology

Small interfering RNA (siRNA) transfection experiments were done using double-stranded RNA synthesized by Dharmacon (Chicago, IL). A nontargeting siRNA (scramble) was used as control. Cells were transfected with Dharmafect 1 (Dharmacon) according to the manufacturer's instructions. Nucleofection standard protocols have been established.⁹

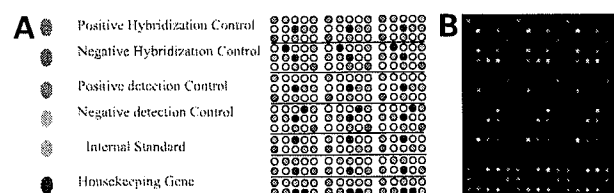


Figure 1. A, design of DualChip human ABC. The array included 49 genes (including 8 housekeeping genes). Each capture probe is spotted in triplicate. Three complete sub-arrays are schematically drawn. Six different internal standards are placed in different areas for normalization. B, fluorescence image of the DualChip human ABC hybridized with cDNA obtained from mRNA isolated from one T-ALL sample.

⁷ <http://www.dtp.nci.nih.gov>

⁸ <http://www.eppendorf.com/microarrays/>

⁹ <http://www.amaxa.com/jurkat.html> and <http://www.amaxa.com/jurkatcells.html>

Table 1. Expression of ABC transporters in 21 clinical samples of T-ALL

HUGO nomenclature	Synonyms	Mean value*	Mean CV of samples [†]	Mean CV of triplicates [‡]	Number of tumors in which the gene was detected [§]
ABCA1	ABC1	1,127	NA	NA	0
ABCA2/3	ABC2/3	3,709	53, 79	12, 64	14
ABCA4	ABC10	684	NA	NA	1
ABCA5	ABC13	306	NA	NA	0
ABCA6		85	NA	NA	0
ABCA7	ABCX	21,062	74, 68	4, 78	21
ABCA8		80	NA	NA	0
ABCB1/4	MDR1/4	77	NA	NA	0
ABCB2	TAP1	28,358	86, 77	4, 18	21
ABCB3	TAP2	2,539	NA	NA	8
ABCB6	ABC14	2,215	NA	NA	10
ABCB7	ABC7	6,930	102, 48	6, 9	21
ABCB8	ABC22	3,118	56, 85	15, 46	14
ABCB9	ABC23	1,220	NA	NA	0
ABCB10	M-ABC2	5,649	99, 11	12, 47	17
ABCB11	BSEP/SPGP	60	NA	NA	0
ABCC1	MRP1	21,325	86, 53	5, 96	21
ABCC2	MRP2	108	NA	NA	0
ABCC3	MRP3	657	NA	NA	0
ABCC4	MRP4	634	NA	NA	0
ABCC5	MRP5	3,985	74, 69	11, 98	15
ABCC6/8/9	MRP6/7/8	576	NA	NA	0
Kir6.1/6.2		738	NA	NA	0
ABCC7	CFTR	102	NA	NA	0
ABCC10	MRP7	4,871	85, 25	14, 11	17
ABCD1	ALD	2,912	67, 06	22, 23	9
ABCD2	ALDR	1	NA	NA	0
ABCD3	PMP70	7,449	121, 15	10, 77	18
ABCD4	PXMP1L	242	NA	NA	0
ABCE1	RNS4I	9,106	132, 1	12, 92	19
ABCF1	ABC50	13,157	101, 5	7, 18	21
ABCF2	ABC28	4,133	110, 78	14, 11	15
ABCF3	ABC25	3,384	81, 37	14, 31	13
ABCG1	ABC8	1,808	NA	NA	6
ABCG2	BCRP	283	NA	NA	0
IMPT		2394	NA	NA	5

NOTE: NA, nonapplicable.

*Mean value (signal intensity) from 21 samples of T-ALL cancer expression. The values were calculated according to the Materials and Methods, and normalized by the ratios obtained from the internal standards and housekeeping genes comparing each sample with a reference sample.

[†] Coefficient of variance was obtained by calculating the mean of the CV of each sample and is expressed as a percentage.[‡] The mean CV of triplicates is expressed as the mean of the CV of the triplicates of all experiments.[§] The gene is considered as detected in the tumor if the signal intensity was >2.5-fold over the signal intensity of negative controls.

The effect of ABCA2 and ABCA3 expression silencing on drug-induced resistance was analyzed as followed: 143B cells were seeded in 12-well plates at 100,000 cells/well 24 hours before being transfected with Dharmafect 1 for 24 hours with 20 nmol/L of ABCA2 siRNA, 50 nmol/L of ABCA3 siRNA, or with 50 nmol/L of ABCA2 + 50 nmol/L of ABCA3 siRNAs or equivalent treatment with scramble siRNA. Twenty-four hours posttransfection, media were refreshed and cells were incubated with 0.3 µg/mL of doxorubicin, 0.03 µg/mL of methotrexate, or 0.01 µg/mL of vinblastine. ABCA2 and ABCA3 mRNA level and the effect of drug treatment on transfected cell viability were measured, respectively, by quantitative reverse transcription-PCR and MTT assay 72 hours posttransfection.

Statistical Methods

COMPARE Analysis. The sulforhodamine B assay for the determination of drug sensitivity in 60 cell lines from the NCI panel were reported previously (19). The IC₅₀ values for drugs are included in the Standard Agents Database of the Developmental Therapeutics Program of the NCI.¹⁰ The mRNA expression values of 60 cell lines of ABCA2 and ABCA3 transporter genes (represented by each of three different clones with individual GenBank accession numbers) were selected from the NCI's database. The

¹⁰ <http://dtp.nci.nih.gov>

mRNA expression was determined by microarray analysis as reported previously (20, 21). The microarray data of ABC transporters was confirmed by real-time reverse transcription-PCR analyses (22). COMPARE analysis was done to produce rank-ordered lists of cytotoxic compounds. The COMPARE methodology has been previously described in detail (23). Briefly, every standard drug of the NCI's database is ranked for the similarity of its IC_{50} values to the mRNA expression for the *ABCA2* or *ABCA3* transporters. To derive COMPARE rankings, a scale index of correlation coefficients (R values) was created. In the standard COMPARE method, greater mRNA expression in cell lines correlate with greater IC_{50} values, e.g., increase in drug resistance.

MTT Assay. We used Student's t test to calculate the significance of the differences in the cell viability between the treated and untreated cells.

Computation of the Theoretical Reference

A theoretical reference has been computed to obtain a global mean of the tumors by averaging all the tumor data. This reference was used as a comparison mean for each tumor and allowed the attainment of a gene expression profile with higher or lower expressed genes in each tumor.

The construction of this global mean was done in two steps. First, each array was normalized to a chosen reference array (sample no. 213). The normalization factors were obtained by the internal standards and housekeeping genes spotted on the arrays as described by de Longueville et al. (16). This normalization step levels up the intensities of the array data to a chosen one. A global weighted mean of the arrays was then computed using these factors for all data obtained at a given photomultiplier scan setting.

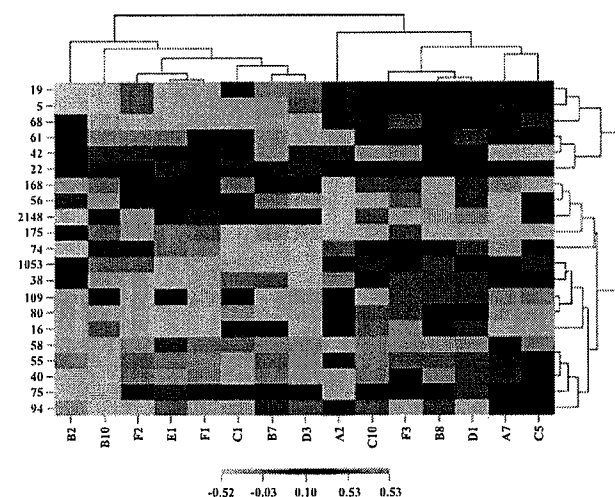


Figure 2. Cluster analysis done using CIMMiner software (http://discover.nci.nih.gov/nature2000/tools/upload_s.jsp) on the genes which were detected on the microarray (38 ABC transporters, 1 cationic transporter, and 2 ATP-sensitive potassium channels) in 21 T-ALL samples. The data used for the clustering were the ratios obtained for one gene in each sample compared with the mean value in the 21 samples.

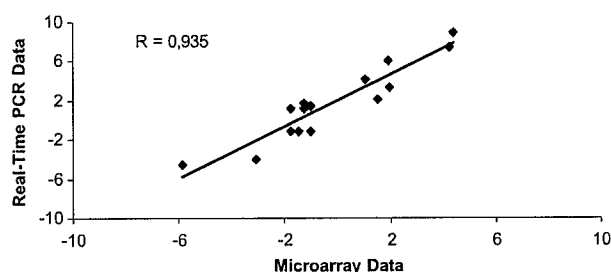


Figure 3. Validation of microarray data by real-time reverse transcription-PCR. The expression of seven ABC transporter genes was assayed by reverse transcription-PCR in three randomly selected poor response tumors compared with one tumor with good response to chemotherapy. The relative expression levels measured by real-time reverse transcription-PCR were first corrected for the values to the α -tubulin gene. The results of the real-time reverse transcription-PCR were correlated with the microarray-based expression values (Spearman's rank correlation test). The values are presented as the ratios of ABC gene expression in poor response tumor versus good response tumor.

Results

Expression Profiling of Clinical Samples

First, we analyzed the expression of ABC transporters in 21 clinical ALL samples by means of the DualChip Human ABC low-density microarray. The data from one representative experiment of an ALL sample (no. 42) is shown in Fig. 1B. Reliability and the reproducibility between assays were assessed by repeating the experiments with several replicates depending on the amount of RNA available. The mean coefficient of variance (CV) for the triplicates inside an array was 11.33%, calculated on the quantitative detected genes in 43 arrays. For seven samples, we could perform triplicate arrays and the mean CV of all detected genes was 14.58%.

In order to detect an increase or decrease in the expression of the different ABC transporters in the different tumors, we compared the expression of individual ALL with the mean values obtained in the 21 T-ALL (global mean; Table 1) and 4 of them were very abundant. The other ABC transporters were not significantly detected.

Among the 15 genes which were well-expressed, some were expressed rather constantly in all the tumors, whereas others were expressed very variably. This is reflected in the SD of the means. Given the fact that the CV for the triplicates of each probe in one array and for experiments performed in triplicates were $<15\%$, we concluded that the variation of the means of all the tumors really reflects the variation of expression between the tumor samples.

The expression values of high- and low-regulated ABC transporters were subjected to cluster analysis. As shown in Fig. 2, the cluster tree clearly separates up- or down-regulated ABC transporters (red and blue color codes, respectively; black represents nondetected genes) from other ABC transporters whose expression was within the global mean. The cluster analysis in Fig. 2 illustrates that *ABCA2/3* was frequently overexpressed in several samples, indicating that *ABCA2/ABCA3* may be of relevance for ALL. Moreover, we can observe that *ABCA2/3* was

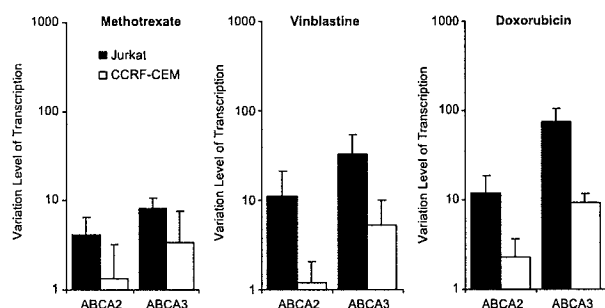


Figure 4. Expression of *ABCA2* and *ABCA3* in Jurkat and CCRF-CEM cells after treatment with cytostatic drugs (methotrexate, vinblastine, and doxorubicin). After 72 h of incubation, the expression measured by real-time reverse transcription-PCR is given in relation with the expression of untreated cells.

significantly expressed in 14 tumors with a mean intensity value of 3,709 (Table 1). Correlations between the overexpression of ABC transporters and the clinical data were not conclusive. We checked for correlations with initial response to therapy, overall and relapse-free survival, age, sex, WBC count, and percentage of leukemic cells at presentation (data not shown).

To validate the microarray data, we selected seven ABC transporter genes and analyzed their expression in four ALL samples (three from patients showing a poor response to treatment and one from a patient showing a good response to treatment as reference) taken randomly from the 21 samples by real-time reverse transcription-PCR. As shown in Fig. 3, the mRNA expression of seven ABC transporters obtained by real-time reverse transcription-PCR of three tumor samples (poor response patient) compared with a reference

from a good response patient, correlated significantly with the corresponding values obtained by microarray analysis at a significance level of $P = 1.53 \times 10^{-7}$ and a correlation coefficient of $R = 0.935$ (Pearson's correlation test).

Induction of *ABCA2* and *ABCA3* Expression by Drug Treatment

Because the hybridization signals for *ABCA2/A3* were present in most T-ALL samples, we checked whether these two genes might be involved in the response of T-ALL to cytostatic drugs. We treated CCRF-CEM and Jurkat T-ALL cell lines with methotrexate (1 and 0.1 $\mu\text{g}/\text{mL}$), vinblastine (0.001 and 0.1 $\mu\text{g}/\text{mL}$), or doxorubicin (1 and 0.1 $\mu\text{g}/\text{mL}$). After 72 hours of incubation, we quantified the mRNA expression of *ABCA2* and *ABCA3* in treated and untreated cell samples by real-time reverse transcription-PCR. The ratios of mRNA expression of treated and untreated cell aliquots are shown in Fig. 4. Although all three drugs led to an increase of *ABCA3* expression in Jurkat and CCRF-CEM cells, a significant increase of *ABCA2* expression was only observed in Jurkat cells. These results indicate that both genes may be involved in the drug response of T-ALL cells.

Down-Regulation of *ABCA2/A3* Expression by RNA Interference

To confirm the possible implications of *ABCA2* and *ABCA3* in drug resistance, we inhibited their expression by RNA interference (RNAi) and tested for the change in cell resistance to the drugs. Because CCRF-CEM and Jurkat cells were difficult to transfect with RNAi, we decided to use another cell line to address this question: the osteosarcoma 143B cell line. The control panel in Fig. 5A shows that the transfection agent Dharmafect, scramble sequences, methotrexate (0.03 $\mu\text{g}/\text{mL}$), vinblastine (0.01 $\mu\text{g}/\text{mL}$), or doxorubicin (0.3 $\mu\text{g}/\text{mL}$) applied alone

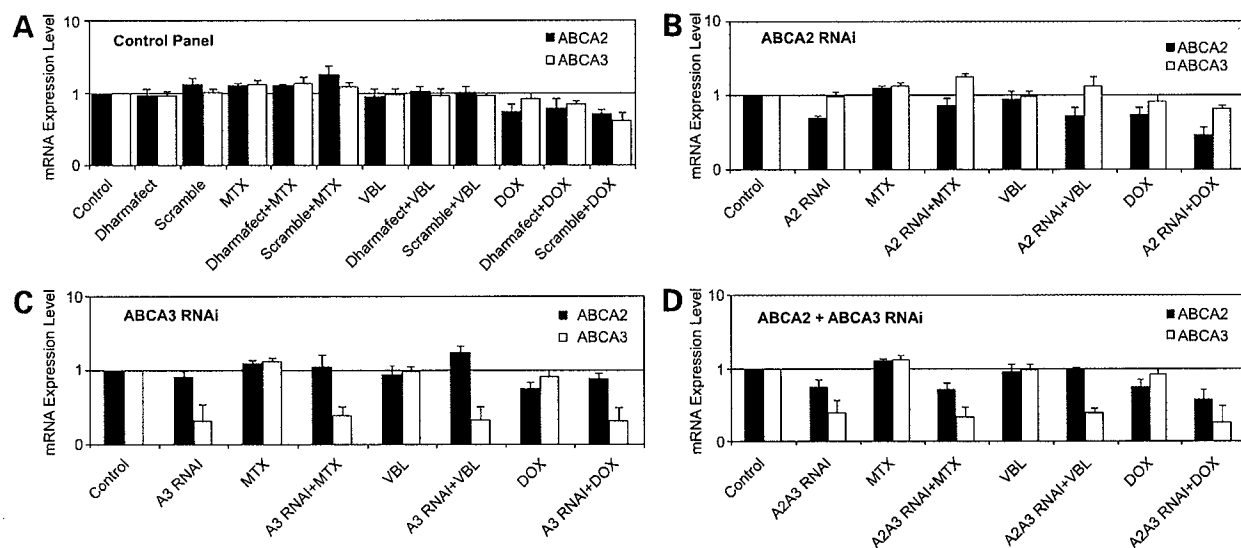


Figure 5. Sensitivity of 143B cells to methotrexate, vinblastine, and doxorubicin after transfection with siRNA for *ABCA2* and/or *ABCA3* and after treatment with nontargeting siRNA (scramble). Cell survival was measured by MTT-assays. The results are given as the percentage of cell survival compared with untreated samples (control).

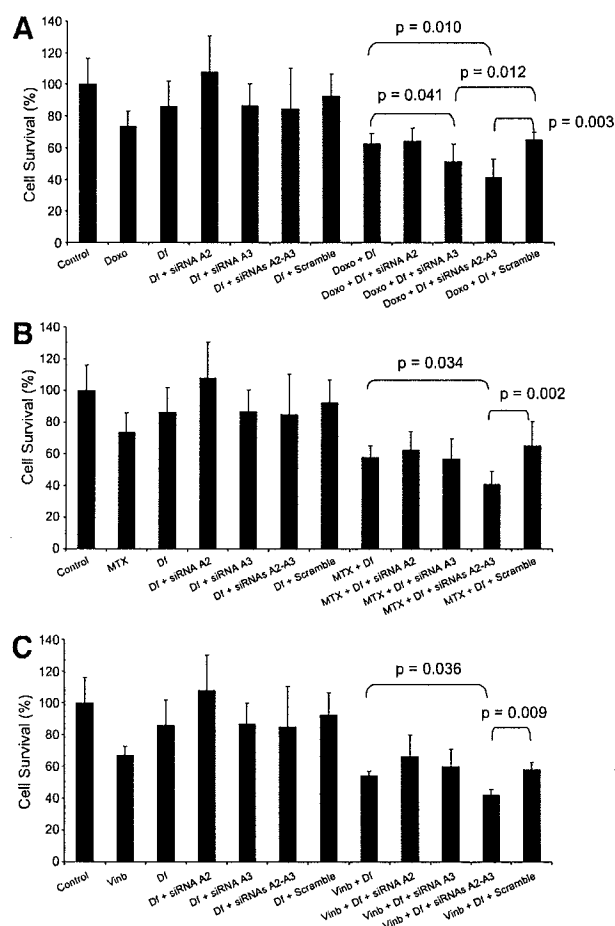


Figure 6. Transfection of *ABCA2* and *ABCA3* genes measured by real-time PCR in 143B cells transfected with *ABCA2* and/or *ABCA3* siRNA and with scramble siRNA. The results are given as ratios to the expression in untreated samples.

or in combination had no or only a minimal effect on the mRNA expression of *ABCA2* or *ABCA3*. As a next step, we applied *ABCA2* RNAi alone or in combination with drugs. *ABCA2* RNAi down-regulated mRNA expression of *ABCA2*. If *ABCA2* RNAi was combined with methotrexate, vinblastine, or doxorubicin, an up-regulation of *ABCA3* expression was observed for the first two drugs, which was not visible after treatment with *ABCA2* RNAi alone (Fig. 5B). A comparable effect was found after treatment with *ABCA3* RNAi. As expected, the application of RNAi alone reduced the expression of *ABCA3* but not *ABCA2*. Cotreatment with *ABCA3* RNAi plus methotrexate and vinblastine resulted in an increased expression of *ABCA2* (Fig. 5C). If RNAi for both *ABCA2* and *ABCA3* were applied together, an increased inhibition of expression of *ABCA2* and *ABCA3* was observed, either with or without additional drug treatment ($P < 0.05$; Fig. 5D). These results indicate that the RNAi transfection specifically inhibits the targeted gene and that a combination of *ABCA2* or *ABCA3* siRNA with methotrexate and vinblastine leads to a compensation effect.

Next, we used the MTT assay to measure the effects of RNAi on cell viability. The transfection agent Dharmafect and scramble sequences were only minimally cytotoxic ($<20\%$ reduction in cell viability), as were methotrexate, vinblastine, and doxorubicin in the concentrations used (0.03, 0.01, and 0.3 $\mu\text{g}/\text{mL}$, respectively; Fig. 6A–C). RNAi for *ABCA2* or *ABCA3* applied alone did not induce or only minimally induced cytotoxicity. A combination of the three drugs plus RNAi for *ABCA2* or *ABCA3* resulted in a moderate increase in cytotoxicity compared with each agent applied alone, except for the combination of doxorubicin and *ABCA3* siRNA that showed a significant increase in cytotoxicity ($P = 0.041$ compared with transfection reagents; $P = 0.012$ compared with scramble). If RNAi for both *ABCA2* and *ABCA3* were applied together and combined with drugs, a stronger cytotoxic effect was observed (Fig. 6A–C). These differences were statistically significant when we compared these latter results with transfection reagents, and was highly significant when compared with scramble sequences (Fig. 6A–C).

COMPARE Analysis

Because RNAi experiments showed that *ABCA2* and *ABCA3* play a role in the resistance to methotrexate, vinblastine, and doxorubicin, we investigated the role of these ABC transporters in drug resistance in more detail. We correlated the constitutive mRNA expression of these two ABC transporters in 60 cell lines of the NCI panel with the IC_{50} values for compounds included in the NCI's Standard Agent Database. The microarray and drug response data can be found in the NCI database.¹¹ The two transporter genes are represented by each of three different clones with individual GenBank accession numbers. One clone of the *ABCA2* gene and one clone of the *ABCA3* gene have been analyzed twice in microarray experiments. They have different pattern identifier numbers (Table 2). Drugs for which IC_{50} values correlated with microarray-based mRNA expression of these ABC transporter genes with COMPARE correlation coefficients of $R < 0.2$ were not considered further. IC_{50} values of drugs correlating with at least two microarray data sets are listed in Table 2. This approach was applied to explore which compounds may be involved in drug resistance mediated by *ABCA2*, *ABCA3*, or both. The results indicate that the IC_{50} values of several compounds correlated with both *ABCA2* and *ABCA3* genes (rapamycin, tricinibine phosphate, a 5,12-naphthacenedione derivative, cytoxan, 4'-deoxydoxorubicin, α -2'-deoxy-6-thioguanosine, pibenzimol, and O⁶-methylguanine). Other drugs correlated either with *ABCA2* alone (pentostatin, mitotane, hexamethylene bisacetamide, didemnin B, tamoxifen, and teroxirone) or *ABCA3* alone (lomustine and flavone acetic acid). This indicates that both ABC transporters might have an overlapping but not an identical substrate spectrum.

¹¹ <http://dtp.nci.nih.gov>

Discussion

Many studies have been done on the clinical relevance of ABC transporters. Thus far, most of these studies analyzed one or a small group of ABC transporters, e.g., *ABCB1/MDR1*, *ABCC1/MRP1*, and *ABCG2/BCRP*. For most other members of this gene family, the clinical relevance has still not been examined. In the present study, we used a novel low-density DNA microarray for the simultaneous expression analysis of 38 ABC transporters, which we have recently developed (12). The present study is the first one to describe a microarray-based detection of ABC transporter expression in ALL.

One of the positive features of low-density microarray is the good reproducibility and the specific validation that was done for the group of genes detected by the chip (12, 16, 17). This validation is particularly important in a group of genes that shows as much homology as the family of ABC transporters (12, 22). The reproducibility of the arrays is well illustrated by the very low CV of the quantitative detected genes which was <15% in the triplicate spots of the same arrays and in the triplicate arrays done on the same sample.

Besides the four ABC transporters which were highly expressed, *ABCA7*, *ABCB2*, *ABCC1*, and *ABCF1*, we also observed that *ABCA2/A3* was overexpressed in most of the analyzed ALL. These results suggest that *ABCA2* and *ABCA3* may be of importance for ALL. *ABCA2* has been shown to confer mitoxantrone resistance and to transport estramustine (24, 25). Furthermore, daunorubicin and

mitoxantrone are translocated by *ABCA3* (26, 27). Treatment of HL60 leukemia cells with cantharidin, an investigational natural product, induced *ABCA3* expression (28). Yasui et al. (29) found that a number of drug-resistant cancer cell lines showed higher copy numbers of the *ABCA3* gene and a stronger expression of the gene compared with the drug-sensitive parental cell lines. *ABCA3* is located at intracellular membranes (30). It does not confer a "classical" drug efflux across cell membranes but rather seems to be involved in the intracellular sequestration and vesicular drug transport (27, 30). Based on these reports, we hypothesized that *ABCA2* and *ABCA3* may be relevant for drug resistance in T-ALL.

To investigate this hypothesis, induction experiments were done. The T-ALL cell lines, CCRF-CEM and Jurkat, were treated with methotrexate, vinblastine, or doxorubicin. All three drugs led to an increase of mRNA expression of *ABCA3* in both cell lines, whereas a significant increase of mRNA expression of *ABCA2* was only observed in the Jurkat cell line. This indicates that these two transporters may contribute to the resistance of T-ALL to these drugs. These results are in accordance with the reports of the role of *ABCA2* and *ABCA3* in the resistance to mitoxantrone, daunorubicin, and estramustine (24–27).

The RNAi experiments of the present investigation provide evidence for the involvement of both ABC transporters in drug resistance. Because CCRF-CEM and Jurkat cells could not be transfected with sufficient efficacy in our

Table 2. Possible drug substrates of *ABCA2* and *ABCA3* as determined by COMPARE analysis of microarray-based mRNA expression and IC₅₀ values for compounds extracted from the Standard Agent Database of the NCI

NSC no.	Compound	Gene (GenBank accession no) [Pattern identifier]					
		ABCA2 (AB028985) [GC28724]	ABCA2 (AB028985) [GC54846]	ABCA2 (AA748768) [GC49299]	ABCA3 (U78735) [GC38193]	ABCA3 (U78735) [GC97635]	ABCA3 (H51436) [GC12872]
Compounds correlating with <i>ABCA2</i> and <i>ABCA3</i>							
NSC 226080	Rapamycin		X		X	X	X
NSC 280594	Triciribine phosphate	X	X		X	X	
NSC 357704	5,12-Naphthacenedione derivative	X	X		X	X	
NSC 26271	Cytosan	X	X		X		X
NSC 267469	4'-Deoxydoxorubicin	X	X				X
NSC 71851	α-2'-Deoxy-6-thioguanosine	X	X				X
NSC 322921	Pibenzimol HCl				X	X	X
NSC 37364	O ⁶ -Methylguanine			X		X	X
Compounds correlating with <i>ABCA2</i>							
NSC 218321	2'-Dexoycoformycin, pentostatin	X	X				
NSC 38721	Mitotane	X	X				
NSC 95580	Hexamethylene bisacetamide		X	X			
NSC 325319	Didemnin B	X	X				
NSC 180973	Tamoxifen	X	X				
NSC 296934	Teroxirone	X	X				
Compounds correlating with <i>ABCA3</i>							
NSC 79037	Lomustine (CCNU)				X	X	X
NSC 347512	Flavone acetic acid					X	X

NOTE: See <http://dtp.nci.nih.gov> for more details.

study, we decided to use 143B cells, which are more easily transfectable. We do not know why the two leukemia cell lines could not be transfected because nucleofection standard protocols have been established (see Materials and Methods).

Treatment with *ABCA2* or *ABCA3* RNAi resulted in a partial down-regulation of *ABCA2* or *ABCA3* mRNA, respectively. An unexpected observation was that cotreatment of *ABCA2* RNAi plus methotrexate and vinblastine led to an up-regulation of *ABCA3*. Vice versa, *ABCA3* RNAi plus these two cytostatic drugs increased the expression of *ABCA2*. This observation was made in three independent experiments. A possible explanation could be that methotrexate and vinblastine are transported by both ABC transporters and that the down-regulation of one transporter was at least compensated by the up-regulation of the other one. Such a mechanism would allow coping with cytotoxic challenge more efficiently.

As a strategy to further explore the possible role of *ABCA2* and *ABCA3* transporters as drug transporters, COMPARE analyses were done with compounds included in the NCI's Standard Agent database and these two ABC transporters, whose mRNA expression in 60 NCI cell lines has been determined by microarrays (20, 21). The COMPARE computation provided a list of drugs that could be considered as substrates for *ABCA2* and *ABCA3*. Although such correlation analysis does not provide evidence for a compound being a true ABC transporter substrate, this strategy can be used to generate testable hypotheses. Our aim was, however, not to provide a complete list of possible substrates for ABC transporters but to obtain information that the *ABCA2* and *ABCA3* transporters could be considered as candidate drug transporters. The results of the COMPARE analysis reinforce the use of the DualChip human ABC as a tool to detect ABC transporter-associated drug resistance. Interestingly, the IC_{50} values of several compounds have been found to correlate with mRNA expression levels of both *ABCA2* and *ABCA3*. Other compounds correlated only with *ABCA2* or only with *ABCA3*. This indicates that the spectrum of possible substrates is overlapping, but not identical. This result fits with the RNAi experiments in the present investigation. The full spectrum of substrates has yet to be explored to compare the multidrug resistance phenotypes of *ABCA2* and *ABCA3* with those of the established multidrug resistance-mediating transporters, *ABCB1/MDR1*, *ABCC1/MDR1*, *ABCC2/MDR2*, and *ABCG2/BCRP*.

Among the established multidrug resistance-conferring ABC transporter genes, *ABCB1/MDR1* was not overexpressed in the T-ALL samples of the present study, and underscores the debate regarding the role of the *ABCB1/MDR1* gene in the drug resistance of T-ALL. In contrast to acute myeloid leukemia, in which the role of the *ABCB1/MDR1* gene for drug resistance of tumors and prognosis of patients is widely accepted (31, 32), data for ALL are conflicting (7, 9, 32–40). It is, therefore, reasonable to propose that ABC transporters other than *ABCB1/MDR1* may be more decisive for treatment response and prognosis

of T-ALL. In our investigation, the *ABCG2 (BCRP)* transporter was not significantly detected whereas *ABCC1 (MDR1)* was highly expressed. Again, the contribution of these three ABC transporters to the treatment outcome of T-ALL patients has been shown by some, but not all authors (8, 36, 41–46), leaving the prognostic relevance of these ABC transporters open to discussion.

In the present investigation, we found several ABC transporters to be down-regulated in some T-ALL samples whereas being rather highly expressed in the overall samples. Among them, *ABCF1* and *ABCB2* are interesting genes because they are well expressed in the tumors but are found to be relatively underexpressed in several other tumors. The biological relevance of this finding is unknown.

Whereas overexpression is compatible with the function of ABC transporters as drug efflux pumps, down-regulation raises the possibility that ABC transporters, i.e., *ABCF1*, might act as influx transporters for cytostatic drugs. Indeed, we found that *ABCF1* expression correlated inversely with the IC_{50} values for 6-mercaptopurine in the NCI cell line panel. Hence, high *ABCF1* expression was associated with drug sensitivity (data not shown). Unlike the majority of ABC proteins, which are membrane-associated transporters, *ABCF1* associates with the ribosome and probably functions in mRNA translation (47). A possible role for *ABCF1* in drug sensitivity deserves further investigation.

Acknowledgments

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